Toll-like receptor 3 ligand attenuates LPS-induced liver injury by down-regulation of toll-like receptor 4 expression on macrophages

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Edited by Philippa Marrack, National Jewish Medical and Research Center, Denver, CO, and approved September 29, 2005 (received for review June 2, 2005)

This study demonstrates that pretreatment with polyinosinic-polycytidylic acid (poly I:C) significantly decreased the mortality and liver injury caused by injection of lipopolysaccharide (LPS) in the presence of D-galactosamine (D-GalN) in C57BL/6 mice. Depletion of natural killer, natural killer T, and T cells did not change the protective effect of poly I:C on LPS/D-GalN-induced liver injury *in vivo*. However, depletion of macrophages abolished LPS/D-GalN-induced fulminant hepatitis, which could be restored by adoptive transfer of macrophages but not by transfer of poly I:C-treated macrophages. Treatment with poly I:C down-regulated the expression of the toll-like receptor 4 (TLR4) on macrophages and reduced the sensitivity of macrophages (Kupffer cells and peritoneal macrophages from C57BL/6 mice, or RAW264.7 cells) to LPS stimulation. Poly I:C pretreatment also impaired the signaling of mitogen-activated protein kinases and NF-κB induced by LPS in RAW264.7 cells. Blockade of TLR3 with a TLR3 antibody abolished poly I:C down-regulation of TLR4 expression and LPS stimulation of TNF-α production in RAW264.7 cells. Taken together, our findings suggest that activation of TLR3 by its ligand, poly I:C, induced LPS tolerance by down-regulation of TLR4 expression on macrophages.

Lipopolysaccharide (LPS)/D-galactosamine (D-GalN)-induced hepatitis is a well established model of liver injury mediated by macrophages (1–3). Upon stimulation by LPS, macrophages secrete proinflammatory cytokines, including IL-1, IL-6, IL-12, and TNF-α (4). Among these factors, TNF-α is the terminal mediator of hepatic apoptosis and organ failure. TNF-α-induced hepatocyte apoptosis has been identified as an early and possibly causal event during LPS/D-GalN-induced liver failure (2, 5, 6). Furthermore, TNF-α-induced neutrophil transmigration in the later stages of liver injury has been shown to be a critical step in hepatocyte necrosis in this model. Hence, massive hepatocyte apoptosis induced by TNF-α from macrophages is the dominant mechanism of liver injury in this model (7–9).

Polyinosinic-polycytidylic acid (poly I:C) is an artificial mimic of viral RNA and induces immune responses similar to a viral infection (10). Poly I:C has been used with a self-peptide to induce chronic, severe pancreatitis in MRL/+ mice (11, 12). Previous studies in mice have demonstrated that poly I:C caused a mild liver injury and inhibited liver regeneration (13, 14). The action of poly I:C is believed to be mediated by targeting of toll-like receptor 3 (TLR3) (15).

TLRs are a family of proteins that recognize specific patterns of microbial components, especially those from pathogens, and regulate innate and adaptive immune responses (16). The TLR family now consists of at least 13 members (TLR1–TLR13) in the mouse genome, whereas 11 TLRs have been found in humans (16, 17). The ligands for most TLRs have been identified. TLR4 has been identified as a receptor for LPS, and TLR4-deficient mice are hyporesponsive to LPS, demonstrating that TLR4 is a critical receptor for LPS signaling (18, 19).

In this study, we demonstrated that TLR3 activation induced by poly I:C prevented LPS-induced fulminant hepatitis by down-regulation of macrophage TLR4 expression.

Materials and Methods

Animals. Male C57BL/6 mice (6–8 weeks old, weighing 20–24 g) were obtained from the Shanghai Experimental Center, Chinese Science Academy, Beijing, and maintained at an animal facility under pathogen-free conditions. The handling of mice and experimental procedures were conducted in accordance with experimental animal guidelines.

Cell Line. Mouse macrophage cell line RAW264.7 was obtained from the Shanghai Cell Bank. Cells were cultured in RPMI medium 1640 in 10% FBS within a humidified incubator containing 5% CO_2 at 37°C and passed every 2–3 days to maintain logarithmic growth.

Reagent. Poly I:C, LPS (*Escherichia coli*, 0111:B4), and D-GalN were purchased from Sigma and reconstituted in PBS. All reagents including poly I:C and depleting antibodies were endotoxin-free to the limits of detection of the Tachypleus Amebocyte Lysate (TAL) assay (Fuzhou Xinbei Biochemical Industrial, Fuzhou, China).

Experimental Protocol. Hepatic damage was induced in mice by an i.p. coinjection of LPS (5 μg/kg of body weight) and D-GalN (400 mg/kg of body weight), each in a solution of 200 μl per dose. Poly I:C was dissolved in PBS for a final concentration of 1 mg/ml and administered in a single i.p. dose (7.5 μg/g of body weight) at various time points before subsequent coinjections with LPS and D-GalN. For *in vitro* experiments, RAW264.7 cells were treated with either LPS (100 ng/ml), poly I:C (50 μg/ml), or PBS.

Isolation of Peritoneal Macrophage. Peritoneal macrophages were isolated from C57BL/6 mice. Mice were i.p.-injected with 2 ml of 4% thioglycolate. Three days after thioglycolate injection, cells in the peritoneal exudates were isolated by washing the peritoneal cavity with ice-cold Hank's balanced salt solution. Collected cells were incubated for 4 h, and adherent cells were taken as peritoneal macrophages.

Assay for Serum Alanine Aminotransferase (ALT) and Aspartate Aminotransferase (AST). To assay for serum ALT and AST levels, mice were anesthetized with ether and bled from the eye. Serum (50

Conflict of interest statement: No conflicts declared.

This paper was submitted directly (Track II) to the PNAS office.

Abbreviations: poly I:C, polyinosinic-polycytidylic acid; D-GalN, D-galactosamine; TLR, toll-like receptor; ALT, alanine aminotransferase; AST, aspartate aminotransferase; NK, natural killer; MAPK, mitogen-activated protein kinase; MyD88, myeloid differentiation factor 88.

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μl) was mixed with 0.5 ml of ALT or AST assay solution (Shanghai Rongsheng, Shanghai, China) and then measured in a spectrophotometer following the supplier's protocol.

Hematoxylin and Eosin Staining. For histological analysis, liver tissue was fixed in 10% neutral-buffered formalin and embedded in paraffin. Sections of 5-μm thickness were affixed to slides, deparaffinized, and stained with hematoxylin and eosin to determine morphologic changes.

Measurement of Serum Cytokine Levels. The serum samples were kept at −20°C until ready for cytokine measurement. Levels of TNF-α and IL-12 were measured by using commercially available ELISA kits from R&D Systems.

Cell Depletion. A dose of 50 μl of anti-AsGM1 antiserum (Wako Pure Chemical, Osaka) diluted in 200 μl of pyrogen-free PBS was injected i.v. into mice 1 day before treatment to deplete natural killer (NK) cells. Anti-NK1.1 mAb (PK136), anti-$\alpha\beta$TCR mAb (H57–597), and anti-$\gamma\delta$TCR mAb (UCT-13D5) were obtained from the American Type Culture Collection from partially purified hybridoma culture supernatant by ammonium sulfate precipitation. Mice were given three injections of the indicated mAb (50 μg per mouse) i.p. on days 2, 1, and 0 before subsequent injections. This protocol resulted in a ≥90% decrease in the number of indicated cells.

Macrophages were eliminated in mice by injection of gadolinium chloride ($GdCl_3$), purchased from Sigma. Macrophages were depleted *in vivo* 24 h after i.v. injections of $GdCl_3$ (10 mg/kg of body weight) (20, 21).

Cell Transfer. Peritoneal macrophages were collected from C57BL/6 mice and washed twice in PBS. The degree of contamination by other cells was minimal. Macrophage transfer was performed as described (22). Briefly, under ether anesthesia, peritoneal macrophages (1×10^7 cells) stimulated with PBS or poly I:C (50 μg/ml) suspended in 100 μl of pyrogen-free PBS were injected i.v. into the mice without macrophages, and recipient mice received coinjections of LPS and D-GalN i.p.

RT-PCR Analysis. RNA was extracted from liver tissue by using Trizol Reagent (Invitrogen). Cellular RNA (1 μg) was used for cDNA synthesis. For real-time PCR, we used the specific kit from Applied Biosystems. PCR primers for detecting mRNA for TLR4, TLR3, and β-actin were synthesized by Shanghai Genecore Biotechnologies, Shanghai, China. Primer sequences were as follows: β-actin, sense, 5′-GGA CTC CTA TGT GGG TGG CGA GG-3′, antisense, 5′-GGG AGA GCA TGC CCT CGT AGA T-3′; TLR4, sense, 5′-GCT ATC TGT GAG CGT GTA T-3′, antisense, 5′-ACG GCA ACT TGG ACC TG-3′; and TLR3, sense, 5′-AAG AGG GCG GAA AGG TG-3′, antisense, 5′-GAA GCG AGC ATT TAC TA-3′.

Flow Cytometric Analysis. mAbs were used in this study, including FITC-conjugated anti-mouse F4/80 (Caltag, South San Francisco, CA). Cell surface expression of TLR4 was assessed by using phycoerythrin-conjugated mAb against murine TLR4 (eBioscience, San Diego). Rat serum anti-mouse TLR3 was used to block TLR3 signaling (eBioscience).

After blocking with anti-FcrR (eBioscience), cells were incubated with saturating amounts of the indicated fluorescence-labeled mAbs at 4°C for 30 min in darkness and then washed twice. The stained cells were analyzed by using a FACScalibur flow cytometer (Becton Dickinson), and the data were processed with WINMDI2.8 software. All nonviable cells were excluded by forward scatter, side scatter, and propidium iodide gating. For the intracellular cytokine assay, cells were fixed, permeabilized, and stained for intracellular TLR4 with a Cytofix/Cytoperm plus kit (Pharmingen).

Immunocytoplasmic and Histological Staining. A standard immunocytoplasmic staining protocol was used in this study (23). Briefly, RAW264.7 cells were cultured in a chamber slide (Nalge), washed with PBS, and air-dried. Slides were fixed with methanol for 30 min at −20°C and then stained with the phycoerythrin-conjugated rat anti-mouse mAbs TLR4 or isotype control rat IgG2a (eBioscience) for 24 h at room temperature.

Western Blotting. Cellular extracts were prepared as described (24). Fifty micrograms of total protein was mixed in Laemmli loading buffer, boiled for 5 min, and then subjected to SDS/PAGE. After electrophoresis, proteins were transferred onto nitrocellulose membranes (Schleicher & Schuell, BA85), and blotted against primary Abs overnight at 4°C. Membranes were washed with 0.05% (vol/vol) Tween 20 in PBS (pH 7.6) and incubated with a 1:2,500 dilution of horseradish peroxidase-conjugated secondary Abs (Promega) for 60 min at room temperature. Protein bands were visualized by ECL reaction (Pierce).

Statistical Analysis. Results were analyzed by using Student's *t* test or ANOVA where appropriate. All data are expressed as the mean ± SEM. P values < 0.05 were considered significant.

Results

Poly I:C Pretreatment Prevented LPS/D-GalN-Induced Fulminant Hepatitis. Coinjection with LPS and D-GalN in mice induced severe liver injury and a high mortality in a short time. The dose of coinjection (5 μg/kg LPS, 400 mg/kg D-GalN) was selected to induce live injury. In addition, the dose of poly I:C (7.5 μg/g of body weight) was chosen because it did not induce liver injury and could activate lymphocytes (25). In this experiment, poly I:C was administered at various time points before coinjection of LPS and D-GalN. As shown in Fig. 1*A*, the effect of poly I:C pretreatment on LPS/D-GalN-induced hepatitis was time-dependent. Pretreatment with poly I:C 6 h before LPS/D-GalN injection markedly prevented LPS/D-GalN-induced mortality, whereas pretreatment 24 h before the coinjection did not. Other time points for poly I:C treatment to the coinjection was also experimented: the effect of 2–6 h before coinjection was similar, yet 6 h was the best time point. The protective effect of poly I:C was also confirmed by results revealing that poly I:C pretreatment inhibited elevation of serum ALT and AST levels in LPS/D-GalN-treated mice (Fig. 1*B*). Furthermore, pathology analysis showed that poly I:C pretreatment completely inhibited hepatocyte destruction induced by coinjection of LPS and D-GalN (see Fig. 5, which is published as supporting information on the PNAS web site).

Because TNF-α is a critical mediator of liver injury after injection of LPS/D-GalN (2, 5, 6), we postulated that the protective effect of poly I:C on LPS/D-GalN-induced hepatitis was mediated by inhibiting elevation of serum TNF-α levels. Administration of LPS/D-GalN significantly increased serum TNF-α levels, which peaked at 90 min postinjection (data not shown). Therefore, we measured serum TNF-α levels 90 min after the injection of LPS/D-GalN. As shown in Fig. 1*C*, injection of poly I:C did not elevate serum TNF-α levels, whereas administration of LPS/D-GalN markedly induced elevation of serum TNF-α levels. Pretreatment with poly I:C attenuated LPS/D-GalN-mediated induction of TNF-α. In contrast, injection of either LPS/D-GalN or poly I:C elevated serum IL-12 levels, and poly I:C administration induced IL-12 levels even greater than LPS/D-GalN administration. Cotreatment with poly I:C and LPS/D-GalN did not additively or synergistically induce elevation of serum levels of IL-12 (Fig. 1*C*). Collectively, these results

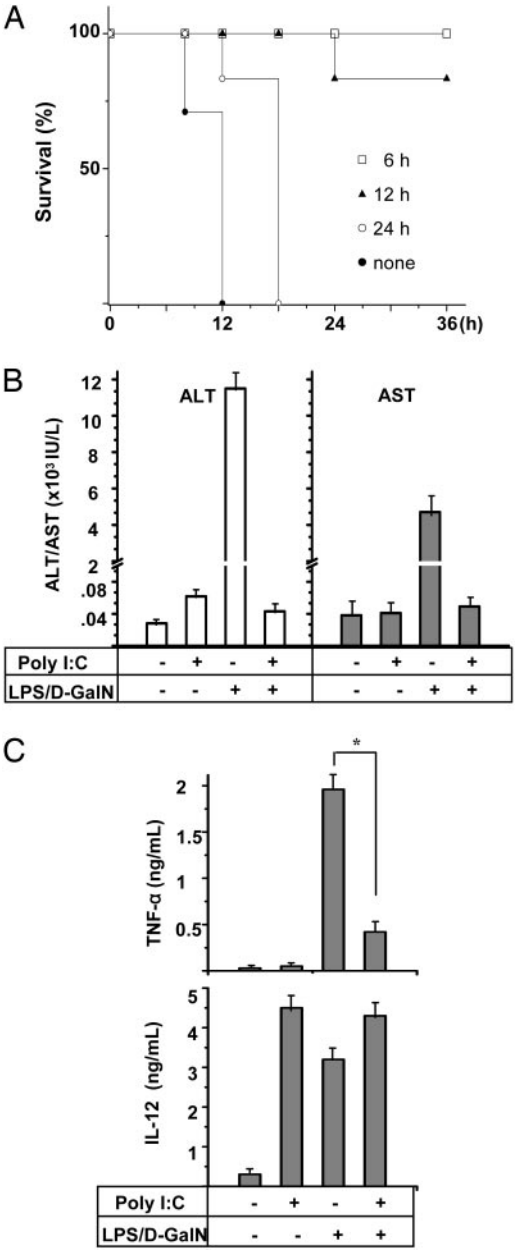

Fig. 1. Pretreatment with poly I:C attenuated LPS-induced acute liver injury in C57BL/6 mice. (*A*) Poly I:C (7.5 μg/g) was injected i.p. for 6, 12, and 24 h before coinjection with a lethal dose of LPS (5 μg/kg) and D-GalN (400 mg/kg). Survival rate was measured after coinjection. (*B*) Poly I:C was i.p.-injected into mice 6 h before LPS/D-GalN injection. Serum samples were collected 6 h after coinjection, and serum transaminase activity was determined. (*C*) Serum samples were collected at 90 min and 2 h post-LPS/D-GalN injection for measuring TNF-α and IL-12. Data in *B* and *C* are expressed as the mean $\pm$ SEM (n = 5–7). *, $P < 0.05$ in comparison with LPS/D-GalN alone.

indicated that poly I:C pretreatment strongly inhibited LPS-induced liver injury in mice.

Poly I:C Acted on Macrophages Directly and Down-Regulated TLR4 Expression on Macrophages. LPS/D-GalN-induced lethality was triggered by macrophages. To determine whether lymphocyte subpopulations, including $\alpha\beta$ T cells, $\gamma\delta$ T cells, NK T cells, and NK cells, were involved in the protection by poly I:C of LPS/D-GalN-induced liver injury, mice were pretreated with antibodies including anti-$\alpha\beta$ TCR, anti-$\gamma\delta$ TCR, anti-NK1.1, and anti-AsGM1 mAbs. Results of these experiments revealed that lymphocytes did not participate in the course: depletion of these lymphocytes did not influence the protective effect of poly I:C pretreatment to LPS/D-GalN-induced lethality. Next, cell transfer experiments were performed to determine whether the protective effect of poly I:C was mediated by targeting macrophages. As shown in Fig. 2*A*, depletion of macrophages by $GdCl_3$ abolished LPS/D-GalN-induced elevation of serum ALT and AST levels. Adoptive transfer with peritoneal macrophages treated with PBS restored LPS/D-GalN-induced liver injury in macrophage-depleted mice, whereas poly I:C-treated peritoneal macrophages failed to restore such injury. These results suggested that macrophages became hyporesponsive to LPS after poly I:C treatment.

It has been reported that down-regulation of TLR4 is one possible mechanism contributing to LPS-induced endotoxin tolerance (26). Therefore, we hypothesized that poly I:C induction of LPS tolerance may be also mediated by down-regulating TLR4 expression on macrophages. As shown in Fig. 2*B*, poly I:C injection at some time down-regulated TLR4 mRNA expression in liver tissues compared with that in the liver of PBS-treated mice. We also detected the expression of other TLRs (e.g., TLR2 and TLR9) in liver by poly I:C treatment; there was almost no change in them (data not shown). The expression of TLR4 on the surface of Kupffer cells was also reduced after poly I:C treatment for 6 h *in vivo* (Fig. 2*C*).

Similar results were found in other macrophages *in vitro*. Poly I:C treatment for 6 h down-regulated TLR4 expression on the surface of RAW264.7 cells (Fig. 2*D Left*). FACS analysis showed that intracellular TLR4 expression was also down-regulated in RAW264.7 cells (Fig. 2*D Right*). Moreover, the immunohistological examination demonstrated that poly I:C stimulation greatly decreased the distribution of TLR4 either on the cell surface or intracellularly (Fig. 2*E*).

At the same time, we also examined the *in vivo* (Fig. 2*C*) and *in vitro* (data not shown) effect of poly I:C on expression of TLR4 at 12 and 24 h after treatment. At 12 h the expression of TLR4 was also decreased as compared with the control, and it was higher than the expression at 6 h; whereas at 24 h TLR4 expression was restored. The time course of TLR4 expression after poly I:C stimulation seemed to be in accordance with the effect of poly I:C on the mortality caused by LPS/D-GalN. It is also suggested that the variety of TLR4 is important to the LPS/D-GalN-induced injury.

Pretreatment with Poly I:C Inactivated the LPS-Induced Signaling Pathways. It has been reported that LPS activated a variety of signaling pathways, including mitogen-activated protein kinases (MAPKs) and NF-κB and then induced the production of inflammation cytokines (27, 28). As shown in Fig. 3*A*, treatment with LPS induced activation of these pathways (extracellular signal-regulated protein kinase 1/2, p38 MAPK, Jun N-terminal kinase/stress-activated protein kinase, and inhibitor κB) in RAW264.7 cells. Such activation was markedly attenuated by poly I:C pretreatment.

The effect of poly I:C treatment on LPS-mediated induction of TNF-α by macrophages was examined. Poly I:C pretreatment impaired the ability of RAW264.7 cells to secret TNF-α when stimulated with LPS, but did not affect IL-12 production (Fig. 3*B*).

Blockade of TLR3 Prevented the Down-Regulation of TLR4 Expression on Macrophages Induced by Poly I:C. Expression of TLR3 was detected in macrophages, dendritic cells, and epithelial cells, and poly I:C stimulation could up-regulate TLR3 expression on all of these cells (29, 30). RT-PCR analysis showed that TLR3 mRNA expression was also detected in the liver, peritoneal macrophages from C57BL/6 mice, and RAW264.7 cells (data not shown).

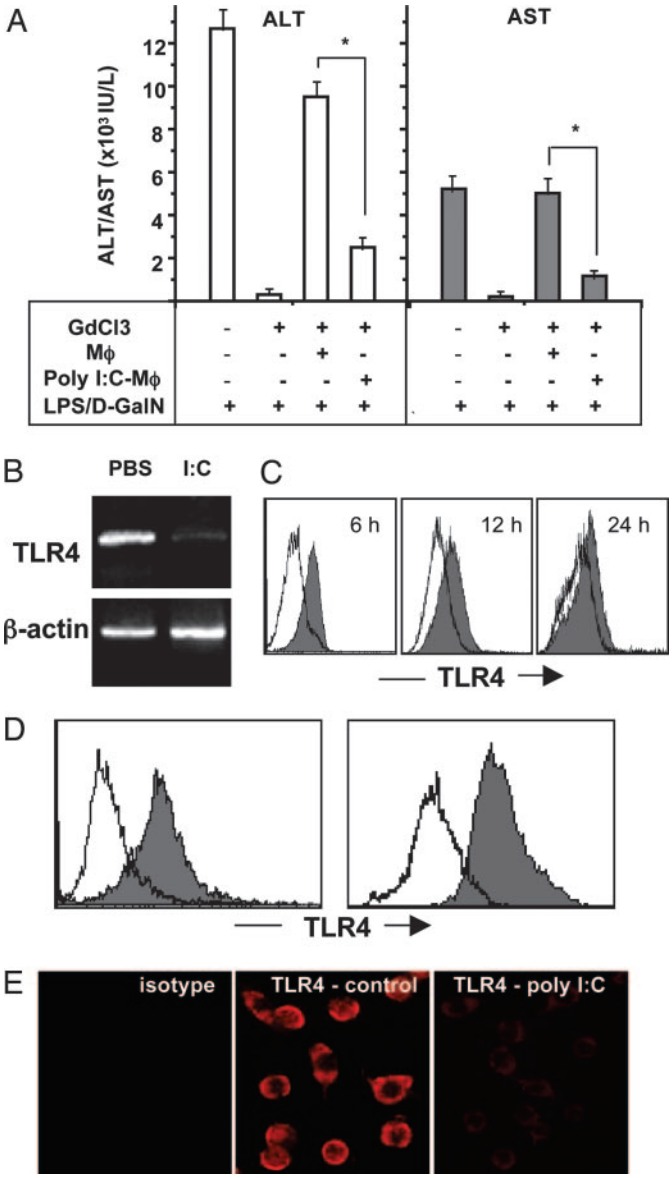

Fig. 2. Poly I:C acted on macrophages directly and down-regulated TLR4 expression on macrophages in this protective process. (*A*) Peritoneal macrophages (1×10^7 cells) were treated with PBS or poly I:C (50 μg/ml) for 6 h, followed by three washes with PBS, and transferred i.v. into macrophage-depleted mice by $GdCl_3$ treatment. After transfer, mice were injected with 5 μg/kg LPS and 400 mg/kg D-GalN. Sera were obtained 6 h later, and ALT/AST levels were measured. Data are expressed as the mean ± SEM of three mice in each group. *, $P < 0.05$. (*B*) The mRNA level of TLR4 was detected by RT-PCR in the liver tissues of C57BL/6 mice treated with PBS or poly I:C for 6 h. I:C was the group of poly I:C stimulation. (*C*) TLR4 expression on the surface of Kupffer cells from C57BL/6 mice treated with poly I:C (7.5 μg/g) or PBS for 6, 12, and 24 h was determined by flow cytometry. Kupffer cells were gated on the $F4/80^+$ population. (*D*) RAW264.7 cells were stimulated with poly I:C (50 μg/ml) or PBS for 6 h. TLR4 expression on the cell surface (*Left*) or intracellularly (*Right*) was examined by flow cytometry. In *C* and *D*, filled histograms represent the PBS-treated group, and the empty histograms are the poly I:C-treated group. (*E*) Immunohistological analysis for detecting the distribution of TLR4 in RAW264.7 cells. RAW264.7 cells stimulated with poly I:C or PBS for 6 h were treated as in *Materials and Methods*. Fluorescence microscope images showed the change of TLR4 expression after poly I:C stimulation.

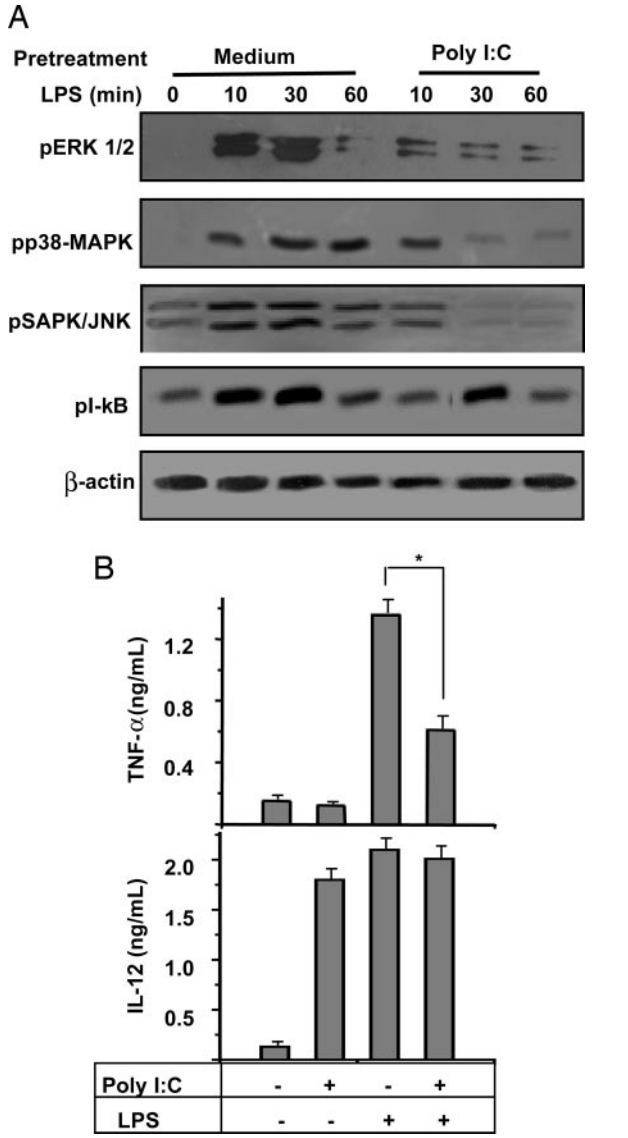

Fig. 3. Pretreatment with poly I:C inactivated the LPS-induced signaling pathways. (*A*) RAW264.7 cells (5×10^6 cells) were pretreated with either buffered medium or poly I:C (50 μg/ml) for 6 h, followed by stimulating with LPS (100 ng/ml) for the indicated time points. MAPK [p38MAPK, extracellular signal-regulated protein kinase 1/2 (ERK1/2), and Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK)] and inhibitor κB (I-κB) phosphorylation was determined by Western blot. (*B*) RAW264.7 cells (1×10^6 cells) were stimulated with poly I:C or media. After 6 h cells were washed twice and then stimulated with LPS. Supernatants were collected 24 h after secondary stimulation for TNF-α and IL-12 evaluation by ELISA. *, $P < 0.05$. The experiments were repeated three times with similar results.

Then we examined whether the down-regulation of TLR4 expression on macrophages is mediated by a TLR3-dependent mechanism. TLR3 antibody was used to block TLR3 exposure on macrophage surfaces. As shown in Fig. 4, treatment with TLR3 antibodies diminished the down-regulation of TLR4 on macrophages induced by poly I:C and restored the ability of secreting TNF-α from RAW264.7 cells stimulated with LPS.

Discussion

Administration of LPS/D-GalN induces fulminant hepatitis in mice. Treatment of mice with D-GalN can lead to a significant increase in sensitivity to the lethal effects of LPS (2, 31, 32). This sensitization is related to the biochemical alterations induced by D-GalN in the hepatocytes (33). In this study, we demonstrated that pretreatment with a nontoxic dose of poly I:C alleviated LPS-induced liver injury. Treatment with poly I:C reduced

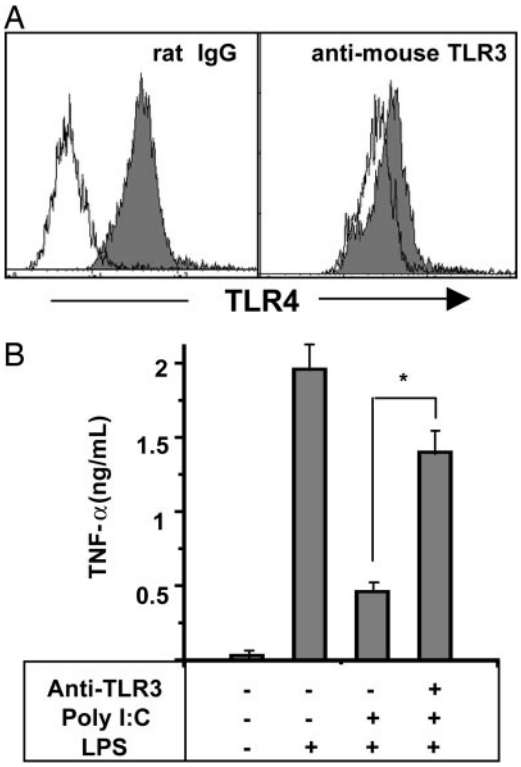

Fig. 4. Blockade of TLR3 prevented the down-regulation of TLR4 induced by poly I:C. RAW264.7 cells (1×10^6 cells per ml) were incubated with anti-mouse TLR3 antibody (1 μl/ml) or rat IgG for 30 min, then treated by poly I:C (50 μg/ml) for 6 h. (*A*) TLR4 expression was analyzed by flow cytometry, then cells were stimulated with LPS (100 ng/ml) for 24 h. Filled histograms represent the PBS-treated group, and empty histograms are the poly I:C-treated group. (*B*) The supernatants were collected for TNF-α evaluation by ELISA. *, $P < 0.05$.

LPS/D-GalN-induced mortality, elevation of ALT/AST levels, liver necrosis, and elevation of serum TNF-α production.

It has been reported that poly I:C activated macrophages, NK cells, and other lymphocyte subpopulations (34, 35). Thus there are two likely mechanisms underlying poly I:C protective processes: (*i*) poly I:C might activate a subpopulation of lymphocytes, which negatively regulated macrophages through cell-to-cell interactions or cytokines secretion; and (*ii*) poly I:C might act on macrophages directly, and thereby induce resistance to LPS. The finding that depletion of NK cells, NK T cells, $\alpha\beta$ T cells, and $\gamma\delta$ T cells did not abolish the protective effects of poly I:C on LPS/D-GalN-induced liver injury suggested that NK, NK T, and T cells were not involved in the observed protective effects. Rather, these results suggested that poly I:C treatment prevented LPS/D-GalN-induced fulminant hepatitis through directly targeting macrophages. Adoptive transfer of PBS-treated macrophages restored LPS/D-GalN-induced liver injury in macrophage-depleted mice, whereas transfer of macrophages treated with poly I:C *in vitro* failed to restore such injury.

It has been reported that preexposure to LPS induced a reduced sensitivity of the host (*in vivo*) or of cultured macrophage/monocyte (*in vitro*) to respond to a subsequent LPS challenge. This phenomenon is termed LPS tolerance or endotoxin tolerance (36). The present findings indicated that poly I:C treatment might down-regulate TLR4 surface expression on macrophage, and the intracellular expression of TLR4 was also decreased after poly I:C stimulation. The reduced TLR4 expression was the major reason for the protection of poly I:C to the D-GalN plus LPS-induced liver injury. To date, the molecular mechanisms of endotoxin tolerance remain to be clearly revolved. Since the discovery of the TLRs as the major receptors for bacterial products, many investigators have focused on changes in the TLR signaling pathways as a mechanism of endotoxin tolerance. TLR4 appears to be the main TLR for LPS, and down-regulation of TLR4 cell-surface expression had been examined as a possible mechanism of LPS tolerance. In macrophages from C57BL/6J mice and RAW264.7 cells, TLR4 expression was markedly decreased by treatment with LPS (19, 26, 36–38). In human or rat, this reduction also existed (39, 40). The conclusion from the above findings is in accord with our results. But there were several published data indicating that endotoxin tolerance was not caused by decreased expression of TLR4 (41, 42), which seems contrary to our results. We think there are three possible differences in our model from the classic LPS tolerance model. First, the target tolerance organ is liver in our model but it is the blood system in LPS tolerance. Second, the primer is poly I:C in our system but it is LPS in LPS tolerance. Finally, D-GalN is used as costimulant in our system but there is no costimulant in LPS tolerance. So the role TLR4/LPS played in the D-GalN plus LPS model is possibly different from LPS administration alone. Moreover, what we focus on in this study is not to explore the mechanisms of LPS tolerance, which is usually to use LPS stimulation alone, but to try to describe that poly I:C protects mice against liver injury induced by D-GalN plus LPS, in which LPS is still a major player but a possibly different actor in LPS administration alone.

This study also showed that poly I:C pretreatment diminished the ability of macrophages to respond to LPS-induced signaling activation and TNF-α secretion. TLR4 activation can lead to the activation of MAPKs and the Rel family of transcription factor NF-κB and induce cells to produce inflammatory cytokines, including TNF-α and IL-12. In the case of TLR4 stimulation by LPS, poly I:C pretreatment diminished the ability of macrophages to respond to LPS-induced secretion of TNF-α and attenuated the activation of MAPKs (p38MAPK, extracellular signal-regulated protein kinase 1/2, Jun N-terminal kinase/stress-activated protein kinase) and NF-κB in macrophages. Taken together, these results indicate that poly I:C pretreatment prevented LPS/D-GalN-induced acute hepatitis by inducing LPS tolerance.

Because poly I:C is the ligand of TLR3 (15), our hypothesis was that poly I:C decreased the TLR4 expression by activating TLR3, and TLR3 signaling down-regulated the TLR4 expression. To test this idea, TLR3 antibody was used to block the TLR3 signaling before poly I:C stimulation. The results indicated that blockade of TLR3 signaling prevented poly I:C down-regulation of TLR4 expression and suppression of TNF-α secretion of macrophages induced by LPS. There have been reports that poly I:C pretreatment leads to impaired signaling in an LPS-induced myeloid differentiation factor 88 (MyD88)-independent pathway (43). LPS-mediated signaling consists of at least two signaling pathways, MyD88-dependent and -independent pathways. MyD88-dependent LPS signaling is crucial for the production of inflammation cytokines (e.g., TNF-α, IL-6, and IL-12), and independent LPS signaling induces the IFN-inducible genes (28, 44). According to the present results indicating a protective effect of poly I:C on LPS/D-GalN-induced liver injury, it is plausible that poly I:C affected the MyD88-dependent LPS signaling in the protective process. However, the possibility that poly I:C weakened the MyD88-independent LPS pathway cannot be excluded. Because poly I:C pretreatment down-regulated the expression of TLR4 suggests that signaling in both MyD88-dependent and -independent pathways might be impaired.

TLRs are crucial for the recognition of invading pathogens. Excessive activation of TLRs may contribute to the pathophysiology of diseases by activating uncontrolled immune responses, including sepsis, immunodeficiencies, and asthma (44, 45). Still, moderate

activation of TLRs can increase the ability of an organism to fight infection. Repeated contact with bacterial components down-regulates TLR expression in intestinal epithelial cells (IECs), and the IEC is in a state of cross-hyporesponsiveness to normal bacterial products (46, 47). The findings presented here suggest that reduction of TLR4 expression promoted the survival of mice suffering from acute hepatitis.

The present findings indicate that the LPS tolerance induced by the TLR3 ligand, poly I:C, was caused by the down-regulation of TLR4 expression in macrophages. Although the actual molecular mechanisms underlying down-regulation of TLR4 expression remained unclear, activation of TLR3 is likely involved because blocking TLR3 with TLR3 antibodies prevented down-regulation of TLR4 expression and suppression of TNF-α secretion by macrophages induced by poly I:C. Some regulators of TLR signaling have been found, e.g., Tollip, Nod2, SIGIRR, and RP105. Cross-talks among the signaling molecules possibly exist in the system.

This work was supported by Natural Science Foundation of China Grants 30125038, 30230340, 30328022, and 30328012; National 973 Knowledge Creation Project Grants 2001CB510009, 2003CB515501, 2004CB520804, and 2004CB518807; and National 863 Hightech Project by Ministry of Science and Technology of China Grants 2002AA216151.

1. Xiong, Q., Hase, K., Tezuka, Y., Namba, T. & Kadota, S. (1999) *Life Sci.* **65,** 421–430.
2. Galanos, C., Freudenberg, M. A. & Reutter, W. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 5939–5943.
3. Freudenberg, M. A., Keppler, D. & Galanos, C. (1986) *Infect. Immun.* **51,** 891–895.
4. Martich, G. D., Danner, R. L., Ceska, M. & Suffredini, A. F. (1991) *J. Exp. Med.* **173,** 1021–1024.
5. Leist, M., Gantner, F., Jilg, S. & Wendel, A. (1995) *J. Immunol.* **154,** 1307–1316.
6. Chosay, J. G., Essani, N. A., Dunn, C. J. & Jaeschke, H. (1997) *Am. J. Physiol.* **272,** G1195–G1200.
7. Kawaguchi, K., Kikuchi, S., Hasegawa, H., Maruyama, H., Morita, H. & Kumazawa, Y. (1999) *Eur. J. Pharmacol.* **368,** 245–250.
8. Kuhnle, S., Nicotera, P., Wendel, A. & Leist, M. (1999) *Biochem. Biophys. Res. Commun.* **263,** 433–438.
9. Szalay, K., Domonkos, A., Kovacs, J., Miko, T., Sass, M., Rassoulzadegan, M., Cuzin, F. & Duda, E. (1999) *Eur. J. Cell. Biol.* **78,** 331–338.
10. Wang, M., Ellison, C. A., Gartner, J. G. & HayGlass, K. T. (1998) *J. Immunol.* **160,** 1098–1105.
11. Qu, W. M., Miyazaki, T., Terada, M., Okada, K., Mori, S., Kanno, H. & Nose, M. (2002) *Clin. Exp. Immunol.* **129,** 27–34.
12. Moriyama, H., Wen, L., Abiru, N., Liu, E., Yu, L., Miao, D., Gianani, R., Wong, F. S. & Eisenbarth, G. S. (2002) *Proc. Natl. Acad. Sci. USA* **99,** 5539–5544.
13. Ochi, M., Ohdan, H., Mitsuta, H., Onoe, T., Tokita, D., Hara, H., Ishiyama, K., Zhou, W., Tanaka, Y. & Asahara, T. (2004) *Hepatology* **39,** 1321–1331.
14. Sun, R. & Gao, B. (2004) *Gastroenterology* **127,** 1525–1539.
15. Alexopoulou, L., Holt, A. C., Medzhitov, R. & Flavell, R. A. (2001) *Nature* **413,** 732–738.
16. Takeda, K., Kaisho, T. & Akira, S. (2003) *Annu. Rev. Immunol.* **21,** 335–376.
17. Tabeta, K., Georgel, P., Janssen, E., Du, X., Hoebe, K., Crozat, K., Mudd, S., Shamel, L., Sovath, S., Goode, J., *et al.* (2004) *Proc. Natl. Acad. Sci. USA* **101,** 3516–3521.
18. Hoshino, K., Takeuchi, O., Kawai, T., Sanjo, H., Ogawa, T., Takeda, Y., Takeda, K. & Akira, S. (1999) *J. Immunol.* **162,** 3749–3752.
19. Poltorak, A., He, X., Smirnova, I., Liu, M. Y., Van, Huffel, C., Du, X., Birdwell, D., Alejos, E., Silva, M., Galanos, C., *et al.* (1998) *Science* **282,** 2085–2088.
20. Fukumura, D., Yonei, Y., Kurose, I., Saito, H., Ohishi, T., Higuchi, H., Miura, S., Kato, S., Kimura, H., Ebinuma, H., *et al.* (1996) *Hepatology* **24,** 141–149.
21. Jankov, R. P., Luo, X., Belcastro, R., Copland, I., Frndova, H., Lye, S. J., Hoidal, J. R., Post, M. & Tanswell, A. K. (2001) *Pediatr. Res.* **50,** 172–183.
22. Rothfuchs, A. G., Kreuger, M. R., Wigzell, H. & Rottenberg, M. E. (2004) *J. Immunol.* **172,** 2407–2415.
23. Eldstrom, J. R., La, K. & Mathers, D. A. (2000) *BioTechniques* **28,** 510–518.
24. Ihara, S., Nakajima, K., Fukada, T., Hibi, M., Nagata, S., Hirano, T. & Fukui, Y. (1997) *EMBO J.* **16,** 5345–5352.
25. Dong, Z., Wei, H., Sun, R., Hu, Z., Gao, B. & Tian, Z. (2004) *J. Hepatol.* **41,** 966–973.
26. Nomura, F., Akashi, S., Sakao, Y., Sato, S., Kawai, T., Matsumoto, M., Nakanishi, K., Kimoto, M., Miyake, K., Takeda, K., *et al.* (2000) *J. Immunol.* **164,** 3476–3479.
27. Medvedev, A. E., Kopydlowski, K. M. & Vogel, S. N. (2000) *J. Immunol.* **164,** 5564–5574.
28. Takeda, K. & Akira, S. (2004) *Semin. Immunol.* **16,** 3–9.
29. Matsumoto, M., Funami, K., Oshiumi, H. & Seya, T. (2004) *Microbiol. Immunol.* **48,** 147–154.
30. Applequist, S. E., Wallin, R. P. & Ljunggren, H. G. (2002) *Int. Immunol.* **14,** 1065–1074.
31. Arvelo, M. B., Cooper, J. T., Longo, C., Daniel, S., Grey, S. T., Mahiou, J., Czismadia, E., Abu-Jawdeh, G. & Ferran, C. (2002) *Hepatology* **35,** 535–543.
32. Mignon, A., Rouquet, N., Fabre, M., Martin, S., Pages, J. C., Dhainaut, J. F., Kahn, A., Briand, P. & Joulin, V. (1999) *Am. J. Respir. Crit. Care. Med.* **159,** 1308–1315.
33. Kurose, I., Miura, S., Higuchi, H., Watanabe, N., Kamegaya, Y., Takaishi, M., Tomita, K., Fukumura, D., Kato, S. & Ishii, H. (1996) *Hepatology* **24,** 1185–1192.
34. Cavanaugh, P. F., Jr., Ho, Y. K. & Bardos, T. J. (1996) *Res. Commun. Mol. Pathol. Pharmacol.* **91,** 131–147.
35. Djeu, J. Y., Heinbaugh, J. A., Holden, H. T. & Herberman, R. B. (1979) *J. Immunol.* **122,** 175–181.
36. Fan, H. & Cook, J. A. (2004) *J. Endotoxin. Res.* **10,** 71–84.
37. Yeo, S. J., Yoon, J. G., Hong, S. C. & Yi, A. K. (2003) *J. Immunol.* **170,** 1052–1061.
38. Qureshi, S. T., Lariviere, L., Leveque, G., Clermont, S., Moore, K. J., Gros, P. & Malo, D. (1999) *J. Exp. Med.* **189,** 615–625.
39. Martin, M., Katz, J., Vogel, S. N. & Michalek, S. M. (2001) *J. Immunol.* **167,** 5278–5285.
40. Medvedev, A. E., Lentschat, A., Wahl, L. M., Golenbock, D. T. & Vogel, S. N. (2002) *J. Immunol.* **169,** 5209–5216.
41. Medvedev, A. E., Henneke, P., Schromm, A., Lien, E., Ingalls, R., Fenton, M. J., Golenbock, D. T. & Vogel, S. N. (2001) *J. Immunol.* **167,** 2257–2267.
42. Frantz, S., Kobzik, L., Kim, Y. D., Fukazawa, R., Medzhitov, R., Lee, R. T. & Kelly, R. A. (1999) *J. Clin. Invest.* **104,** 271–280.
43. Sato, S., Takeuchi, O., Fujita, T., Tomizawa, H., Takeda, K. & Akira, S. (2002) *Int. Immunol.* **14,** 783–791.
44. Cook, D. N., Pisetsky, D. S. & Schwartz, D. A. (2004) *Nat. Immunol.* **5,** 975–979.
45. Toubi, E. & Shoenfeld, Y. (2004) *Autoimmunity* **37,** 183–188.
46. Abreu, M. T., Vora, P., Faure, E., Thomas, L. S., Arnold, E. T. & Arditi, M. (2001) *J. Immunol.* **67,** 1609–1616.
47. Otte, J. M., Cario, E. & Podolsky, D. K. (2004) *Gastroenterology* **126,** 1054–1070.